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THE SUBCELLULAR LOCALIZATION OF THE *neu* PROTEIN IN HUMAN NORMAL AND NEOPLASTIC CELLS

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We have examined the subcellular localization of the *neu* protein by immunohistochemistry and immuno-electron microscopy, associated with immunoblotting of normal and neoplastic tissues with 2 monoclonal antibodies (MAbs). Immuno-electron microscopy clearly reveals that *neu* protein resides only on the lateral plasma membrane of the simple epithelium of the breast and on the plasma membrane of malignant breast cells. It is also found on the membranes of the microvilli and the apical vacuoles of the cells of the proximal convoluted tubule of the kidney. In the cytoplasm, the only immunoreactivity detected with both antibodies was on the membrane of the mitochondrial cristae of normal and malignant cells. Immunoblotting reveals that the molecular weight of the membrane protein is 185 and 155 kDa for the mitochondrial protein. The cell membrane staining pattern can be revealed by light microscopic immunohistochemistry only in malignant cells and is therefore specific for malignancy. The membrane expression in normal cells cannot be visualized in this way. The mitochondrial reactivity appears as a cytoplasmic granular staining when examined under the light microscope. Similar cytoplasmic staining has been described previously in other studies with other antibodies against the *neu* protein and has lead to speculation about its function in normal and malignant cells. However, it is demonstrated in this study that it is not the known *neu*-oncogene product.

The function of the *neu* protein is unknown. Understanding its function may be facilitated by its precise localization in normal and malignant cells and by its subcellular distribution on the apical, lateral or basal part of the cell membrane. Previous studies have demonstrated that the *neu* protein is a 185 kDa membrane protein with tyrosine kinase activity (Schechter *et al.*, 1984; Coussens *et al.*, 1985; Bargmann *et al.*, 1986; Yamamoto *et al.*, 1986). Its gene has been cloned and mapped on chromosome 17 (Coussens *et al.*, 1985; Schechter *et al.*, 1985; Fukushige *et al.*, 1986). The structure of the *neu* protein and its similarity with EGF-receptor suggest that it may be a receptor for an as yet unidentified growth factor. It is a transmembrane protein, with a cell-external ligand binding domain and a cell-internal domain with tyrosine-kinase activity, involved in signal transduction (Coussens *et al.*, 1985). Amplification of the gene has been reported in 10–40% of breast carcinomas (Van de Vijver *et al.*, 1988*a,c*). It is expected to be a plasma membrane protein, but several authors have shown a cytoplasmic reactivity by light microscopy. This cytoplasmic distribution was demonstrated in kidney cells, oral mucosa, urothelium (Gullick *et al.*, 1987), normal breast tissue (Venter *et al.*, 1987; De Potter *et al.*, 1989), and breast carcinoma cells (Berger *et al.*, 1988). Cytoplasmic localization of a membrane-bound receptor could be explained by internalization of the receptor (Smith *et al.*, 1988), but it is difficult to understand why substantial immunoreactivity can be detected in cells with a barely detectable *neu* mRNA level (Kokai *et al.*, 1987).

The aim of this study was to localize this oncogene product in normal, neoplastic and hyperplastic tissues and to look for its subcellular distribution. Therefore, an immunohistochemical investigation, and an immuno-electron microscopical study, associated with Western blot experiments, were carried out.

MATERIAL AND METHODS

Biopsy specimens

Biopsy specimens for light microscopic examination were fixed in 10% formalin for 12 hr and embedded in paraffin at 58°C using routine procedures. Five micron sections were stained with haematoxylin and eosin. The selected types of breast lesions are listed in Table I. Three hundred and thirty breast specimens from 182 patients were investigated. The peroxidase-anti-peroxidase technique was applied (De Potter *et al.*, 1989). All sections were dehydrated and mounted in Merckoglas (Merck, Darmstadt, FRG). Control sections were prepared by omitting the primary antibody. The specificity of the 3B5 antibody was tested by incubation with the primary antibody in the presence of added antigen.

For EM examination, tissues were fixed in 2% formaldehyde (from paraformaldehyde) with a 0.1 M phosphate buffer, pH 7.2. After a rinse in the same buffer, small blocks of tissue were embedded in glycolmethacrylate. Ultra-thin sections were collected on naked Ni-grids. They were floated for 30 min on TBS + 1% BSA, pH 7.6, and then kept overnight at 4°C, the primary antibody being diluted 1/100 for 3B5 and 1/3 for 9G6. The grids, which were held by forceps, were jet rinsed with TBS-BSA and floated for 1 hr on a droplet of goat anti-mouse coupled to 15 nm colloidal gold (Janssen, Beerse, Belgium) diluted 1/20. Finally, the grids were jet-rinsed with TBS-BSA and water. The ultra-thin sections were carbon reinforced and stained for 3 min with saturated uranyl acetate in 50% alcohol and for 2 min with lead citrate.

On 6 micrographs taken from sections labelled with 9G6, the area of the ground cytoplasm and mitochondria, outside the apical zone, was measured with a Mini-mop (Kontron, Basel, Switzerland). The number of gold particles in each area was counted and the relative labelling density was calculated. The ratio of the relative labelling density of mitochondria over that of the cytoplasm—considered as background—was taken as a measure for the specific labelling of mitochondria.

Monoclonal antibodies

3B5: this MAb was raised against a synthetic peptide comprising amino acid residues 1242–1255 of the predicted sequence of the *neu* oncogene product (Van de Vijver *et al.*, 1988*b*). 9G6: this MAb was raised against a murine cell line, transfected with a vector, containing the human *neu* cDNA. It specifically recognizes the *neu* protein (Van de Vijver *et al.*, 1988*b*).

Both MAbs were tested on the SKBR-3 cell line, known to over-express the *neu* protein (Van de Vijver *et al.*, 1988*b*), using an immunogold silver enhancement method (Janssen). Intact cells were incubated with 3B5 and 9G6 antibodies,

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TABLE I – RESULTS FOR THE *neu*-ONCOGENE PRODUCT IN VARIOUS ADULT BREAST TISSUES

Carcinoma patients			Non-carcinoma patients		
Lesion (number)	Results +	Results –	Lesion (number)	Results +	Results –
DCIS (6)	4	2			
Invasive duct-cell carcinoma (99)	23	76			
Normal in the vicinity (78)	0	78	Normal (44)	0	44
Normal at a distance (29)	0	29			
Hyperplasia without atypia (12)	0	12	Hyperplasia without atypia (15)	0	15
Hyperplasia with atypia (7)	0	7	Hyperplasia with atypia (7)	0	7
Apocrine metaplasia (19)	0	19	Apocrine metaplasia (9)	0	9
Lactating groups (2)	0	2	Lactating groups (3)	0	3

+: Staining pattern at the cytoplasmic membrane only is considered as being positive; –: no staining pattern at the cytoplasmic membrane.

washed with TBS-BSA, incubated with gold-labelled goat anti-mouse antibody and silver-enhanced. Only intact 9G6 stained cells showed staining of the cell membrane. A weak staining was obtained with 3B5 after permeabilization of the SKBR-3 cells with Triton X 100 (Fig. 1).

Western blot

The frozen tissue of healthy human kidneys and of positive and negative invasive ductal carcinomas of the breast was pulverized to a fine powder using a microdismembrator (Braun, Melsungen, FRG). The powder was re-dissolved and subsequently centrifuged in a Beckman ultracentrifuge at 1,500, 33,000 and 100,000 *g* respectively. The pellets were placed in a lysis buffer containing 1% Triton X 100 and 1 mM PMSF as protease inhibitor. Electrophoresis run on 5% polyacrylamide/SDS gels was carried out at 60 V for 16 hr. After separation, the proteins were blotted onto a nitrocellulose filter (Schleicher and Schull, Dassel, FRG, BA85: 0.45 μm) or onto a polyvinylidene difluoride microporous membrane (Millipore; 0.45μm) for 24 hr at 50 V/0.5 A in 20% methanol containing 25 mM Tris and 192 mM glycine. The filters were incubated in a NTT buffer (150 mM NaCl–10 nM Tris.HCL, pH 8.0–0.05% Tween 20) at 4°C for 72 hr. The *neu* protein was then visualized by incubating the blot with MAbs 3B5 1/3,000 or 9G6 1/100 in a NTT buffer and with a goat anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis, MO. A-5153) 1/2,000 in a NTT buffer, respectively. After each incubation the blot was washed twice in 200 ml of NTT buffer for 15 min. An additional wash was carried out in a 100 mM Tris.HCL buffer, pH 9.5–5mM $MgCl_2$. Colour development was induced by incubating with BCIP (5-bromo-4-chloro-3-indolyl phosphate; Promega, Madison, WI) and NBT (nitro blue tetrazolium; Promega) substrates in a 5-ml alkaline phosphatase buffer until sufficient specific colour was obtained. The reaction was stopped by washing with a stop buffer (5 mM EDTA–20 mM Tris.HCL, pH 8.0). The blot was subsequently rinsed in distilled water and dried. Nine samples of positive breast carcinoma, 2 samples of negative breast carcinoma, 3 samples of kidney and 2 samples of SKBR-3 cells were investigated. Control blots were carried out by pre-incubating the 3B5 antibody with the free peptide against which it was made.

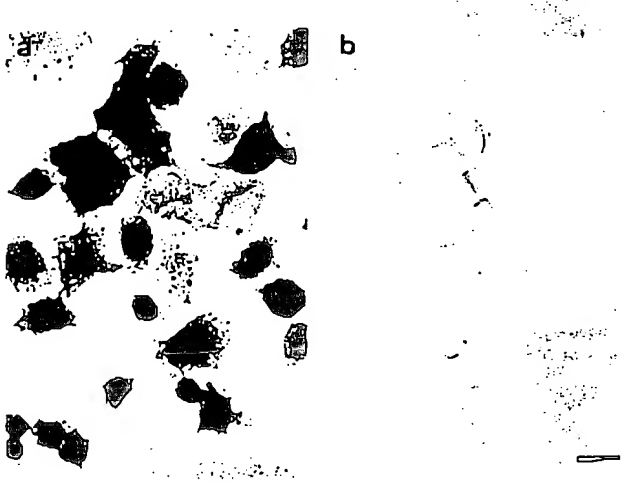

FIGURE 1 – *(a)* SKBR-3 cells stained for the *neu* oncogene product with 9G6. All tumour cells show expression at the plasma membrane. *(b)* SKBR-3 cells stained for the *neu*-oncogene product with 3B5. There is no staining of the cell membranes. Bar = 10μm.

RESULTS

Immunohistochemistry

The results for the various breast lesions, stained with 3B5, are summarized in Table I. In 27 carcinomas, strong staining of the plasma membrane of tumour cells was observed (Fig. 2). Twelve cases showed the *neu*-oncogene product in all tumour cells, the other cases were focally positive. There was no correlation with differentiation of the tumour. Seventy-eight carcinomas (74%), including 2 DCIS, were negative for *neu* membrane staining. Non-malignant breast lesions, normal breast and kidney did not show staining at the plasma membrane. The same results were obtained with 9G6 but staining intensity was weaker and fewer tumour cells were positive.

Several normal cells and breast carcinoma cells showed granular cytoplasmic staining with the antibodies against *neu* (Fig. 3). Experiments in which the primary antibody (3B5 and 9G6) was omitted were consistently negative. Staining of the plasma membrane of the carcinomas and cytoplasmic staining could be completely inhibited by pre-incubating the 3B5 antibody with the synthetic peptide. Pre-incubation of 9G6 with the peptide did not result in inhibition of staining. Comparison between frozen sections and paraffin-embedded material did not reveal differences in the detection of the *neu* oncogene product.

Electron microscopy

1. Neu *localization*

Carcinomas. Carcinoma cells exhibiting a positive and negative plasma membrane pattern for *neu* (see above) were examined at the ultrastructural level. In cases in which well-delineated cell border staining was seen, heavy colloidal gold labelling was observed over the plasma membrane with both antibodies (Fig. 4). There was no staining at the apical membrane of the cells. These cells also had prominent plasma membrane extensions. The reactivity observed by light microscopy is clearly the product of labelling intensity and plasma membrane length. In some of the cases presumed to be negative on light microscopical observation, faint labelling could still be

FIGURE 2 – Invasive duct-cell carcinoma stained for the *neu*-oncogene product with 3B5. All tumour cells show distinct expression at the plasma membrane. Bar = 10μm.

FIGURE 3 – Renal tubules, stained for the *neu*-oncogene product with 3B5 and counterstained with haematoxylin. Cytoplasmic granular staining is observed in the tubular cells. Bar = 10μm.

observed at the ultrastructural level with both antibodies, but a positive signal was lacking in others.

Kidneys. Ultrastructural localization of *neu* in normal tissue was thoroughly studied in the proximal tubules of the kidney. Here a colloidal gold labelling for 3B5 and 9G6 was found over the villi of the brush border and at the membrane, lining the apical vacuoles (Fig. 5). *Neu* protein was excluded from the lysosomes.

Normal breast. A colloidal gold labelling for 3B5, which gave a stronger reaction than 9G6, was seen at the lateral cytoplasmic membrane of the simple epithelia of the ductuli and acini (Fig. 6).

2. Mitochondrial binding of 3B5 and 9G6

Carcinomas. The faint cytoplasmic staining observed by light microscopy with 3B5 in many cells appeared to be due to the binding of antibody to the inner mitochondrial membrane. With 9G6 no measurable binding to mitochondria could be observed in breast carcinoma cells.

Kidneys. In the proximal tubule cells and the cells of the ascending loop of Henle, the mitochondrial cristae were labelled with 3B5 and 9G6 (Fig. 7). In the loop of Henle no other reactivity was observed. With 9G6, labelling was considerably lower, but it was still on average 4.96 (±1.29 standard error of the mean) times above the labelling of the surrounding cytoplasm in areas devoid of apical vacuoles.

Normal breast. The mitochondrial cristae were also labelled with 3B5 and 9G6. The number of mitochondria was considerably lower in these tissues than in the renal tubules.

Western blot

In pellets from positive carcinomas and from kidneys obtained at 33,000 and at 1,500 *g*, a faint staining band was visualized at 185 kDa with both antibodies (Fig. 8). In a 5% SDS-PAGE gels a distinct band was seen at the front. When gradient gels were used, this band gave rise to smaller bands corresponding to molecular weights below 30 kDa. These proteins may be considered as being degradation products due to proteolysis. In the *neu*-negative carcinomas, no reaction could be detected at 185 kDa, but a faint band at 155 kDa from 33,000 *g* pellets was observed (Fig. 9). This band was also obtained with both antibodies in some positive carcinomas and in some kidney samples that exhibited positive mitochondria. It was therefore considered as originating from mitochondria. It was not detected in tumour samples that did not show cytoplasmic staining with immunohistochemistry and containing mitochondria only in small amounts. The supernatant of all tissues at 100,000 *g*, corresponding to the cytoplasmic fraction, did not show a band at 185 kDa. Pre-incubation of the primary antibody with its antigenic peptide made the bands disappear at 185 and 155 kDa and in the front.

DISCUSSION

The precise localization of the *neu* protein may not only contribute to the understanding of its function, but may also provide information on the cellular orientation of the ligand binding sites. Our ultrastructural study shows an accumulation of *neu* protein at the plasma membrane of carcinoma cells that have membrane labelling by light microscopy. Electron micro-

FIGURE 4 – Electron microscopy of carcinoma cells with 3B5 and counterstained with 1% phosphotungstic acid in 1% HCL, revealing both tangentially and cross-cut segments of the plasma membrane. The plasma membrane loops are constantly labelled with colloidal gold particles between neighbouring cells. Bar = 0.25μm.

FIGURE 5 – Electron microscopy of the brush border and apical vacuoles in the proximal tubular cells of the kidney after labelling with 3B5. Colloidal gold particles decorate the villi (upper right) and gold particles can also be observed over the membranes bordering the apical pits and vacuoles (arrowhead). Bar = 0.25μm.

scopical examination also reveals labelling of the lateral membrane of the normal cuboidal epithelium of the ducti and acini of the breast. The Western blot experiments indicate that this protein is the known *neu*-oncogene product, with a molecular weight of 185 kDa. This distribution fits with a putative receptor function, where the ligand is provided by the neighbouring epithelial cells, as could be expected in a paracrine regulatory system. The localization on the cell membrane can be visualized only in malignant cells using immunohistochemistry and light microscopy and at that level it is specific for carcinomas of the breast. This staining occurs in about 26% of invasive breast carcinomas in a series of 182 patients and is not seen in normal and hyperplastic tissues (Gusterson *et al.*, 1988*a,b*; De Potter *et al.*, 1989).

As for normal breast tissue, immunohistochemical investigation of the kidney did not show any membrane reactivity, although the kidney contains large amounts of *neu* mRNA (Kokai *et al.*, 1987). However, EM demonstrates labelling of the membranes of the microvilli and the apical vacuoles of the proximal convoluted tubules, which confirms previous results (Mori *et al.*, 1987). This expression was confirmed by immunoblotting, in which we also detected a 185 kDa protein. The distribution of the *neu* protein on the microvilli and apical vesicles of the renal tubular cells also fits with a receptor function but with a polarity distinct from that of the breast epithelium. In this instance the ligand should be searched for in the primary urine. The above observations localize the *neu*-oncogene product at the plasma membrane of simple epithelia. Yet several authors have also localized *neu* protein in the cytoplasm, using immunohistochemical studies (Gullick *et al.*, 1987; Venter *et al.*, 1987; Berger *et al.*, 1988; De Potter *et al.*, 1989). The cytoplasmic reactivity of antibodies in foetal rat cells has even led to the conclusion that the *neu* protein plays

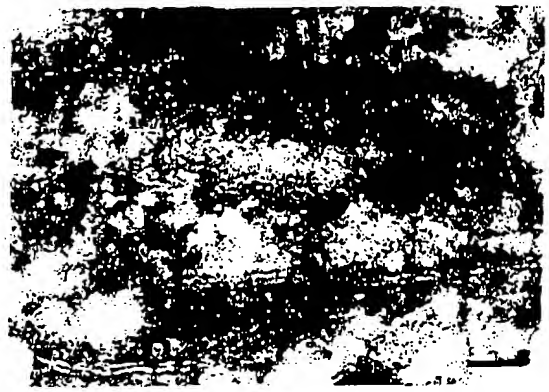

FIGURE 6 – In normal breast cells only a few gold particles were detected over adjoining plasma membranes after electron microscopical labelling with 3B5. Bar = 0.25μm.

an important role in the growth and development of a variety of tissues (Kokai *et al.*, 1987).

We also observed a positive reaction in the cytoplasm of normal renal cells and of some breast carcinoma cells by light microscopy. In EM, this staining pattern appeared to be due solely to binding of antibodies to the membranes of the mitochondrial cristae. With immunoblotting, 2 bands of respectively 185 and 155 kDa were visualized in the kidney samples. The second band was considered to be of mitochondrial origin, as it was the only band detected in negative carcinomas, immuno-electron microscopy showing mitochondrial labelling. This mitochondrial protein is probably a cross-reacting protein, detected with both 3B5 and 90[illegible] may also have

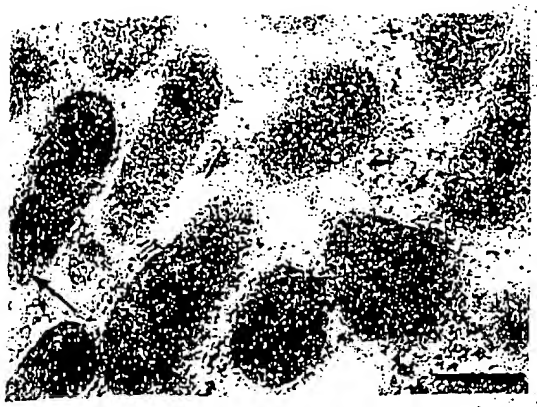

FIGURE 7 – Electron microscopy of mitochondria from proximal tubular cells, treated with 3B5. The mitochondria are heavily labelled with colloidal gold. In places (arrow) it can be recognized that the reactivity is on the mitochondrial cristae. Bar = 0.25μm.

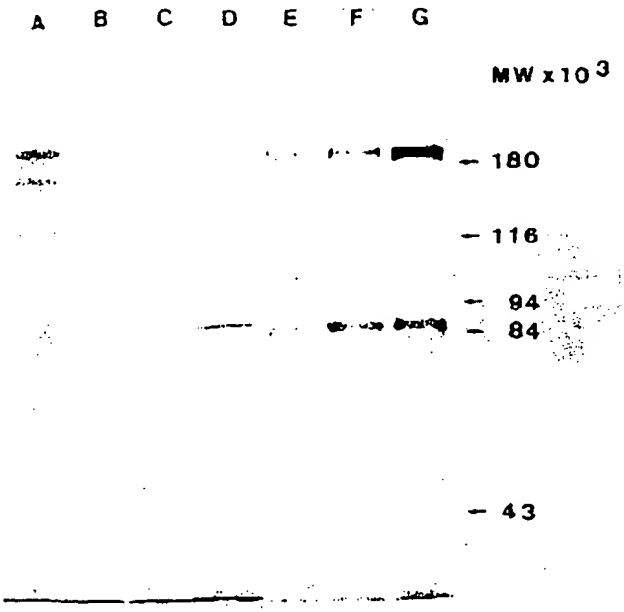

FIGURE 8 – Western blot of renal cortex and of a positive carcinoma with 3B5 on polyvinylidene difluoride filter. Lane A: Faint band at 185 and at 155 kDa disappearing at a decreasing concentration of the homogenate of the kidney in lanes B and C. Lane D: The supernatant of the kidney (A: 100 μg was loaded in 30 μl; B: 50 μg in 30 μl; C: 25 μg in 30 μl; D: 50 μg in 30 μl). Lanes E, F, and G: Band at 185 kDa for increasing concentration of the homogenate of a positive carcinoma (E: 25 μg was loaded in 30 μl; F: 50 μg in 30 μl; G: 100 μg in 30 μl). Lanes D–G: Additional band at 85 kDa.

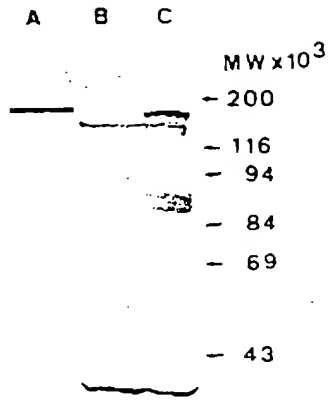

FIGURE 9 – Western blot of SKBR-3 cells and of 2 breast carcinoma specimens with 3B5. Lane A: SKBR-3 cells with a band at 185 kDa (50 μg was loaded in 20 μl). Lane B: Carcinoma with only cytoplasmic reactivity observed by immunohistochemistry and mitochondrial labelling observed by immuno-electron microscopy. Only a band at 155 kDa is observed (100 μg was loaded in 30 μl). Lane C: Carcinoma with membrane staining. Bands at 185 and at 155 kDa are observed (100 μg was loaded in 30 μl).

been the case with several other antibodies against the *neu* protein (Gullick *et al.*, 1987; Kokai *et al.*, 1987; Venter *et al.*, 1987; Berger *et al.*, 1988). Therefore, the observation that the *neu* protein is localized in the cytoplasm of normal cells and the conclusions drawn therefrom should be interpreted with caution. A similar cross-reacting protein is detected by several monoclonal and polyclonal antibodies against CEA (Von Kleist *et al.*, 1972). The "poly-specificity" of MAbs is well known in immunohistochemistry (Andres *et al.*, 1988). Yet it is tempting to hypothesize that the mitochondrial protein might also be coded by the *neu* oncogene, but that it has a different molecular weight and a different destination in the cell. This polymorphism may be explained by post-translational processing of nascent proteins or at the mRNA level by alternative splicing (Breitbart *et al.*, 1987) or the action of different cap sites. Ultrastructural detection of the mitochondrial protein by both antibodies favours this hypothesis. Homology in the external (9G6) and in the internal (3B5) domains, as can be derived from the experiment on the SKBR-3 cell line, means that considerable similarity exists between the mitochondrial and the *neu* proteins.

From our results, we concluded that in normal simple epithelia, *neu* protein resides on the lateral plasma membrane or on the membranes of microvilli and apical vacuoles. This provides an example of 2 distinct cellular localizations of the same receptor function in both instances. Immunoreactivity with a protein other than the *neu* protein was consistently present on the inner mitochondrial membranes, resulting in cytoplasmic staining observed by immunohistochemistry. This finding should be taken into account when interpreting *neu* oncogene over-expression in tumours for diagnostic or prognostic purposes.

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